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Impact of elevated CO₂ concentration on dynamics of leaf photosynthesis in *Fagus sylvatica* is modulated by sky conditions[☆]



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ABSTRACT

It has been suggested that atmospheric CO₂ concentration and frequency of cloud cover will increase in future. It remains unclear, however, how elevated CO₂ influences photosynthesis under complex clear versus cloudy sky conditions. Accordingly, diurnal changes in photosynthetic responses among beech trees grown at ambient (AC) and doubled (EC) CO₂ concentrations were studied under contrasting sky conditions. EC stimulated the daily sum of fixed CO₂ and light use efficiency under clear sky. Meanwhile, both these parameters were reduced under cloudy sky as compared with AC treatment. Reduction in photosynthesis rate under cloudy sky was particularly associated with EC-stimulated, xanthophyll-dependent thermal dissipation of absorbed light energy. Under clear sky, a pronounced afternoon depression of CO₂ assimilation rate was found in sun-adapted leaves under EC compared with AC conditions. This was caused in particular by stomata closure mediated by vapour pressure deficit.

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1. Introduction

Global climate models predict that atmospheric CO₂ concentration ([CO₂]) may increase to as high as 800 μmol (CO₂) mol⁻¹ by 2100. Simultaneously, it is suggested that, on average, frequency of cloud cover will increase with a warming climate, although this prediction varies considerably according to geographic location (IPCC, 2007). Whether rising [CO₂] will cause faster growth and higher photosynthetic carbon storage in C3 plants, and particularly under cloudy sky conditions, remains an open question (Kets et al., 2010; Körner et al., 2005; Leakey et al., 2009).

Under sufficient light intensity, short exposure to elevated [CO₂] leads to an increase in the CO₂ assimilation rate in C3 plants, especially due to depressed photorespiration and enhanced substrate binding by Rubisco (ribulose-1,5-bisphosphate carboxylase/oxygenase) (reviewed in Ceulemans and Mousseau, 1994; Körner

et al., 2005; Norby and Zak, 2011). However, substantial reductions in [CO₂]-enhanced photosynthesis (termed acclimation or down-regulation) may occur over weeks and months of CO₂ treatment and be manifested by a reduction in the Rubisco- and/or RuBP-limited rate of carboxylation (Bernacchi et al., 2003; Davey et al., 2006; Sage, 1990; Urban et al., 2012a).

The genetic constraints of plants, duration of CO₂ enrichment, nutrient supply, and sink strength for carbohydrates have been identified as the main limitations upon a plant's capacity to utilize the additional assimilates (Luo et al., 1999; Norby and Zak, 2011). Increased accumulation of trehalose-6-phosphate and specific saccharides (glucose, sucrose) in leaves may subsequently result in repressed expression of genes transcribing for photosynthetic enzymes (Paul and Pellny, 2003) and decreased stomatal opening in response to light (Lu et al., 1997; Medlyn et al., 2001) over a period of several hours to days, respectively.

Although photosynthetic response to [CO₂] is modified by numerous environmental drivers and by the diurnal dynamics of assimilates accumulation in leaves, studies investigating the diurnal pattern of photosynthesis under ambient and elevated [CO₂] have received only limited attention (e.g. Barták et al., 1999; Bernacchi et al., 2003; Kets et al., 2010; Singsaas et al., 2000;

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Spunda et al., 2005). Previous research on diurnal dynamics has been done under clear sky conditions, within which plants were exposed to saturating irradiances, high temperature, and high vapour pressure deficit (VPD). Such conditions may lead to afternoon depression of photosynthesis, particularly due to stomatal closure (Barták et al., 1999), with subsequent decreases in intercellular $[CO_2]$ and Rubisco carboxylation efficiency, and/or photo-inhibitory constraints (Muraoka et al., 2000; Smirnov, 1993; Zhang et al., 2009). However, the extent of any impact that elevated $[CO_2]$ has on the afternoon decline in photosynthesis remains unclear. Elevated $[CO_2]$ treatment has been shown to ameliorate midday decline in photosynthesis in coniferous *Picea abies* (Spunda et al., 2005), whereas it resulted in more pronounced afternoon decline in photosynthesis compared to ambient concentration in broad-leaved *Populus tremuloides* (Kets et al., 2010).

To the best of our knowledge, no comprehensive study on how elevated $[CO_2]$ influences the diurnal pattern of photosynthesis under cloudy sky conditions had yet been undertaken. Cloud cover boosts the ratio of diffuse to direct solar radiation, and usually it reduces both temperature and VPD. Diffuse radiation has the potential to alter leaf-level (Brodersen et al., 2008) as well as canopy-level CO_2 uptake (Kanniah et al., 2013) compared to an equivalent intensity of direct radiation. Moreover, stomatal responsiveness to elevated $[CO_2]$ may be significantly modified by the actual VPD and/or spectral composition of incident light (Barillot et al., 2010; Heath, 1998), i.e. by environmental drivers which are markedly altered by cloud cover (Urban et al., 2007, 2012b).

To evaluate the benefit of elevated $[CO_2]$ under contrasting sky conditions, we examined diurnal changes in photosynthetic responses of leaves of European beech (*Fagus sylvatica*) trees grown under ambient and elevated $[CO_2]$. Since photons constitute the limiting substrate for photosynthesis under low light intensities, we suggest a positive effect of elevated $[CO_2]$ on carbon uptake only under conditions of high irradiance typical for sunny days. However, excessive irradiance, high VPD, and large accumulation of non-structural carbohydrates in leaves may reduce the stimulation of carbon assimilation near the middle of the day, particularly in sun-exposed leaves.

We tested the hypothesis that differences in the diurnal patterns of microclimate drivers typical for clear and cloudy sky conditions affect the relative impact of elevated $[CO_2]$ on photosynthesis and stomatal conductance. The specific objectives of our study were to investigate (1) diurnal changes in CO_2 assimilation rate and stomatal conductance, (2) changes in Rubisco carboxylation and oxygenation activities *in vivo*, and (3) the role of photosynthetic pigments in the photoprotection of plants during sunny and cloudy days.

2. Materials and methods

2.1. Plants and experimental design

Experiments were carried out in the Beskydy Mountains (Bílý Kríž; 49°30' N, 18°32' E, 908 m a.s.l., in the north-east of the Czech Republic). This area has a cool (annual mean air temperature 6.7 °C) and humid (annual mean relative air humidity 80%) climate with high annual precipitation (the average for 2000–2009 is 1374 mm). Approximately 74% of incident photosynthetic photon flux density (PPFD) is $\leq 500 \mu\text{mol (photons) m}^{-2} \text{ s}^{-1}$ due to frequent cloud cover (Fig. 1).

Eight-year old European beech (*Fagus sylvatica* L.) trees (mean height 2.1 ± 0.45 m) were exposed to ambient ($385 \mu\text{mol (CO}_2\text{) mol}^{-1}$; hereafter AC) and elevated ($700 \mu\text{mol (CO}_2\text{) mol}^{-1}$; hereafter EC) $[CO_2]$ for three growing seasons using glass dome facilities. The dimensions of the glass domes (length \times width) are 10×10 m and the domes are 7 m high at their centres. A system of adjustable lamella-windows and an air-flow climate-control device (with maximum volumetric airflow rate of $4.8 \text{ m}^3 \text{ s}^{-1}$) ensure the maintenance of constant EC and minimize environmental modifications inside the domes. Limited escape of added $[CO_2]$ is achieved using an automated half-closing of windows on the windward side while the remaining windows are left open. The target EC is maintained for ca 75% of the time within the range of $600\text{--}800 \mu\text{mol (CO}_2\text{) mol}^{-1}$ throughout the vegetation season. The air temperature within the domes is maintained within the ambient range ± 1.0 °C for 84% of the time. The domes reduce air humidity and solar radiation

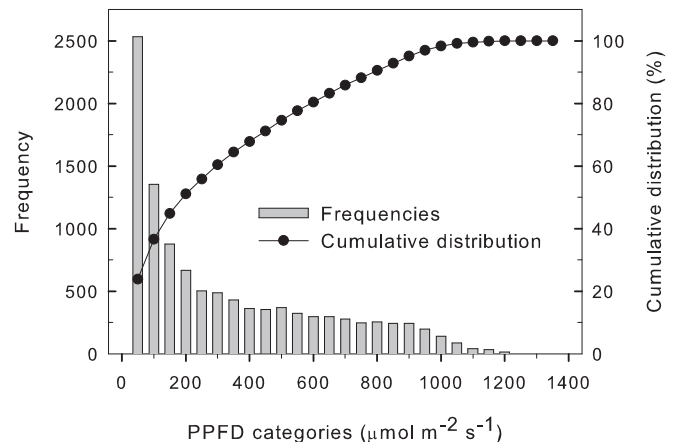


Fig. 1. Frequency of incident photosynthetic photon flux density (PPFD) and its cumulative distribution during the day periods over the vegetation season (May–October) at the experimental site Bílý Kríž (Beskydy Mts., CZ).

by 15% and 26%, respectively, as compared with outside, non-covered area. A detailed technical description of the facility is given in Urban et al. (2001). Sixty-two beech trees per treatment were planted with a spacing of 1.20 m between trees. Plants were grown in the native soil. The geological bedrock is formed by Mesozoic Godula sandstone (flysch type) and is overlain by ferric podzols. Total soil nitrogen was determined to be between 3.5 and 4.2 mg g^{-1} irrespective of CO_2 treatment.

In order to analyse effects of sunny versus cloudy days on CO_2 assimilation processes, two successive periods during the growing season (July) were used for the present analysis. The periods were selected at the same general time of year to minimize the effect of leaf age on physiological parameters. The first two-day period (8–9 July) was cloudy and characterized by a high diffuse index ($DI \geq 0.6$). The second period (22–23 July) was sunny and characterized by a $DI \leq 0.4$ (clear sky) at maximum solar elevation angles. DI is defined here as the ratio between the diffuse and total intensity of photosynthetically active radiation.

Interpretation of the carbon assimilation data was based on the incident PPFD (waveband 400–700 nm). Two quantum sensors (LI-190, Li-Cor, Lincoln, NE, USA) were located above the canopy. To measure daily courses of diffuse PPFD, one quantum sensor was shielded from direct light by shadow rings (CM 121B/C, Kipp & Zonen, Delft, Netherlands).

2.2. Physiological measurements

Leaf-level physiological measurements were done on three representative trees for each $[CO_2]$ treatment. Each replicate consisted of an independent plant selected randomly within the glass dome among those trees of average height, stem diameter, and leaf chlorophyll content. Two fully developed sun-exposed leaves per tree with south or south-west orientation were evaluated and the average from these two measurements used for statistical analyses. All physiological measurements were done at 1.5–2 h intervals from 03:00 (pre-dawn; Central European Time) to 21:30 h (after-sunset). The same leaves were measured on both cloudy and sunny days.

2.3. Chlorophyll fluorescence measurements

The emission of whole-leaf chlorophyll fluorescence was measured at the red band (near 690 nm) using the open version of the FluorCam kinetic imaging fluorometer (Photon Systems Instruments, Brno, Czech Republic), within which the leaves can be exposed to ambient light. Chlorophyll fluorescence signal was measured using short measuring flashes (10 μs pulses with intensity of ca $0.003 \mu\text{mol (photons) m}^{-2} \text{ s}^{-1}$) 800 ms apart. The measuring flashes were generated by two panels of orange LEDs ($\lambda = 620$ nm) and superimposed on the natural light directly at the field site. Subsequently, a strong pulse of white light (1.6 s, $3000 \mu\text{mol (photons) m}^{-2} \text{ s}^{-1}$) was applied to reduce transiently the partially oxidised plastoquinone pool.

Diurnal courses of the quantum yield of photosystem (PS) II photochemistry (Φ_{II}) and quantum efficiencies of regulated ΔpH - and/or xanthophyll-dependent non-photochemical dissipation processes within the PS II antennae (Φ_{NPQ}) were estimated following Savitch et al. (2010) as:

$$\Phi_{II} = (F_M - F_S) / F_M, \quad (1)$$

$$\Phi_{NPQ} = F_S / F_M - F_S / F_M, \quad (2)$$

where F_S is the fluorescence level induced by the actual irradiance, F_M is the maximum fluorescence level observed during 1 s application of saturating pulse

under the actual irradiance, and F_M is the maximum fluorescence level observed during 1 s application of saturating pulse in fully dark-adapted leaves before the sunrise. All chlorophyll fluorescence parameters are presented as averages of whole-leaf fluorescence based on >160,000 leaf pixels.

2.4. Gas exchange measurements

Daily courses of CO_2 assimilation rate (A) and stomatal conductance (G_s) were measured on the intact leaves at their natural orientation using two identically calibrated Li-6400 gas exchange systems (Li-Cor, Lincoln, NE, USA) under conditions of growth CO_2 concentration, ambient irradiance, leaf temperature, and VPD. The air flow rate through the assimilation chamber was maintained at $500 \mu\text{mol s}^{-1}$.

Instantaneous rates of CO_2 assimilation at leaf level (A) were modelled as a general nonrectangular hyperbolic function of incident I :

$$\phi A^2 - (\alpha I + A_{\max})A + \alpha I A_{\max} = 0, \quad (3)$$

where α is the apparent quantum yield, ϕ is a number between 0 and 1 determining the shape of the light–response curve, and A_{\max} is the light-saturated value of A .

In parallel, the relationship between A and intercellular $[\text{CO}_2]$ (C_i) under saturating irradiance ($1400 \mu\text{mol (photons) m}^{-2} \text{s}^{-1}$) was controlled starting at $[\text{CO}_2]$ $350 \mu\text{mol (CO}_2) \text{mol}^{-1}$ in the leaf chamber and decreasing stepwise to $50 \mu\text{mol (CO}_2) \text{mol}^{-1}$. The parameters of the A/C_i response curves were used to derive the maximum Rubisco carboxylation rates *in vivo* ($V_{C_{\max}}$) using the equations of Farquhar et al. (1980). In addition, temperature functions proposed by Bernacchi et al. (2001) for Rubisco-limited photosynthesis were applied to normalize $V_{C_{\max}}$ rates to uniform leaf temperature (25°C).

To evaluate daily dynamics of photorespiration, the leaves were supplied with an atmosphere containing only 2% oxygen. The photorespiration was subsequently quantified as the difference between the photosynthesis rate measured under normal 21% oxygen and that measured under 2% oxygen (Sharkey, 1988).

2.5. Pigment and leaf nitrogen analyses

Approximately 0.1 g of leaves were sampled from the same crown levels at times corresponding to the aforementioned physiological measurements. After determining the leaf fresh mass and planar area (Li-3000A, Li-Cor, USA), the samples were immediately (within 1 min after removing a leaf) frozen in liquid nitrogen and transported to the laboratory.

Chlorophylls (Chls) and carotenoids (Cars) were extracted using 80% acetone and a small amount of MgCO_3 . The clear supernatant obtained after centrifugation at

$480 \times g$ for 3 min was used for spectrophotometric (UV/VIS 550, Unicam, Cambridge, UK) estimation of Chl a, Chl b, and total Cars using the extinction coefficients and equations by Lichtenthaler (1987). The same supernatant was used for the determination of xanthophyll cycle pigments (i.e. A, antheraxanthin; V, violaxanthin; Z, zeaxanthin) by gradient reversed-phase HPLC equipped with a photodiode-array detector UV6000LP (Thermo Fisher Scientific, West Palm Beach, FL, USA). The conversion state of the xanthophyll cycle pigments (de-epoxidation state; DEPS) was calculated according to Demmig-Adams and Adams III (1996) as:

$$\text{DEPS} = [0.5A + Z]/[V + A + Z] \quad (4)$$

The content of non-structural saccharides and starch in leaves was determined using a UV/VIS 550 spectrophotometer (Unicam, Cambridge, UK). The method is based on analysis of the condensation product obtained by heating saccharides or starch with anthrone reagent in acid solution and assessing absorbance at wavelength 625 nm (Yemm and Willis, 1954). Extraction in 95% ethanol followed the method verified for *F. sylvatica* and described in Teslová et al. (2010).

Nitrogen content in the dry mass of leaves was detected using an automatic analyser (CNS-2000, LECO Corporation, St. Joseph, MI, USA) in 0.1 g mixed samples.

2.6. Statistical data analysis

Before the analysis of variance (ANOVA), the data was tested for normality of individual parameters using a Kolmogorov–Smirnov test and homogeneity of variances was tested using Levene's test. For effects of sky conditions, $[\text{CO}_2]$ and time of day, the photosynthetic parameters were analysed using a three-way fixed-effect ANOVA model. For analysis of sky conditions and $[\text{CO}_2]$ effects on content of non-structural saccharides and starch in leaves, a two-way fixed-effect ANOVA was used.

Tukey's post-hoc test ($p = 0.05$) was used to detect significant differences between means within individual sky conditions, $[\text{CO}_2]$ treatments, and time of day. All statistical tests were made using Statistica 9 software (StatSoft, Tulsa, OK, USA).

3. Results

3.1. Microclimate conditions

The measuring campaigns included days that differed primarily in their sky conditions. Although there were transient changes in PPFD (Fig. 2A), DI remained mostly within the ranges of

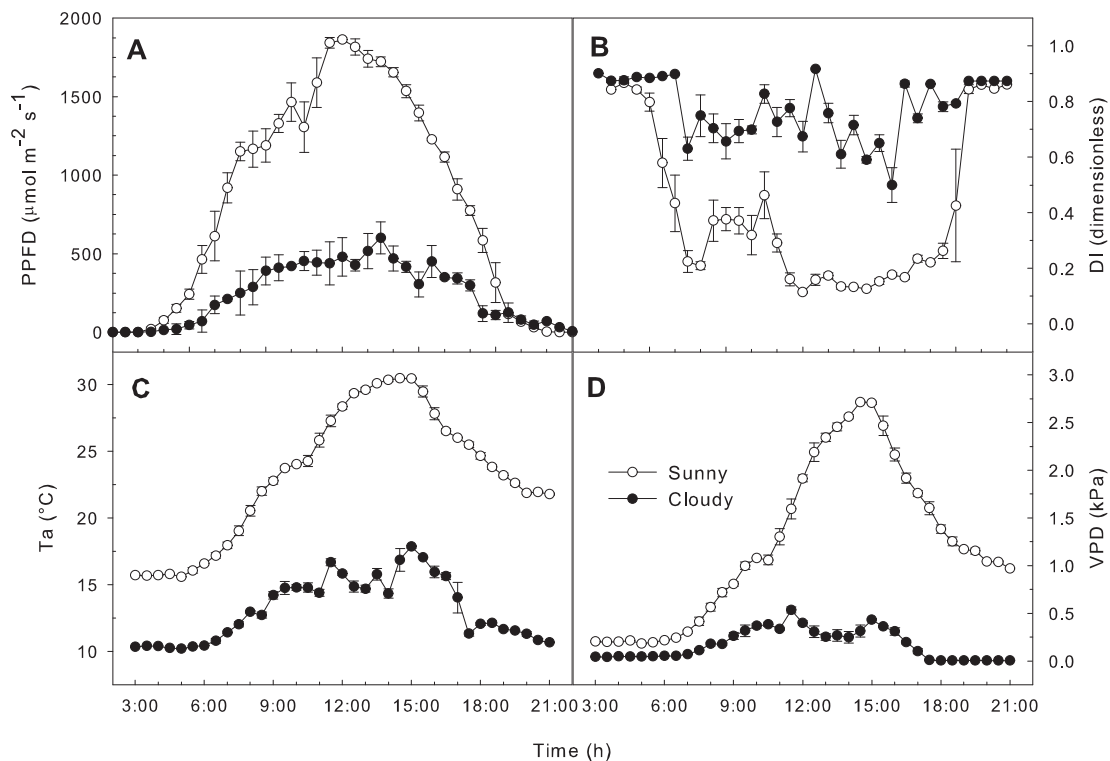


Fig. 2. Diurnal courses of incident photosynthetic photon flux density (PPFD; **A**), diffuse index (DI; **B**), air temperature (T_a ; **C**), and vapour pressure deficit (VPD; **D**) during sunny (open circles) and cloudy (closed circles) days when physiological measurements were carried out. The means (points) and standard deviations (error bars) of 30 min intervals are presented.

Table 1

Mean values \pm standard deviations of physiological parameters of the estimated European beech leaves during cloudy and sunny days: SLM – specific leaf mass, N_{area} – total nitrogen content per unit leaf area, Chl (a + b) – total chlorophyll content per unit area, Chl a/b – ratio between chlorophyll a and b, Cars – total content of carotenoids per unit area, and VAZ – total content of xanthophyll cycle pigments per unit leaf area, DEPS_{max} – maximum de-epoxidation state of the xanthophyll cycle pigments at noon. Identical letters indicate homogeneous groups with statistically non-significant differences ($p > 0.05$). $N = 18–26$.

		SLM	N_{area}	Chl (a + b)	Chl a/b	Cars	VAZ	DEPS _{max}
		$g\ m^{-2}$	$g\ m^{-2}$	$mg\ m^{-2}$	–	$mg\ m^{-2}$	$mg\ m^{-2}$	%
Cloudy	AC	$106 \pm 1.7\ b$	$2.4 \pm 0.2\ a$	$330 \pm 50\ bc$	$3.0 \pm 0.2\ a$	$71 \pm 7\ bc$	$19 \pm 3\ a$	$57 \pm 3.9\ a$
	EC	$115 \pm 2.3\ a$	$2.4 \pm 0.3\ a$	$290 \pm 50\ a$	$3.1 \pm 0.2\ ab$	$64 \pm 8\ a$	$17 \pm 3\ a$	$65 \pm 2.3\ b$
Sunny	AC	$106 \pm 2.3\ ab$	$2.4 \pm 0.2\ a$	$350 \pm 50\ c$	$3.1 \pm 0.1\ b$	$75 \pm 10\ c$	$18 \pm 3\ a$	$71 \pm 4.3\ bc$
	EC	$115 \pm 2.1\ ab$	$2.4 \pm 0.2\ a$	$300 \pm 50\ ab$	$3.2 \pm 0.2\ c$	$69 \pm 7\ ab$	$18 \pm 3\ a$	$76 \pm 4.0\ c$

Table 2

Summary of significance levels (p -values of three-way ANOVA) for the effects of CO₂ treatment, time of day and sky conditions including mutual interactions on the leaf chemical composition (Chl (a + b) – total chlorophyll content per unit area, Chl a/b – ratio between chlorophyll a and b, Cars – total content of carotenoids per unit area, VAZ – total content of xanthophylls per unit leaf area, DEPS – de-epoxidation state of the xanthophyll cycle pigments, NSC – total content of non-structural carbohydrates per unit leaf dry mass, and Starch – total content of starch per unit leaf dry mass). Significant effects and interactions ($p < 0.05$) are indicated in bold.

	Chl (a + b)	Chl a/b	Cars	VAZ	DEPS	NSC	Starch
Sky conditions	0.011	<0.001	0.001	0.435	<0.001	0.001	0.059
Time	<0.001	0.563	0.001	0.086	<0.001	<0.001	<0.001
[CO ₂]	<0.001	<0.001	<0.001	0.054	<0.001	<0.001	<0.001
Sky conditions \times Time	0.114	0.402	0.146	0.041	<0.001	<0.001	0.693
Sky conditions \times [CO ₂]	0.469	0.662	0.813	0.478	0.923	0.803	0.068
Time \times [CO ₂]	0.382	0.419	0.207	0.117	<0.001	0.568	0.523
Sky conditions \times Time \times [CO ₂]	0.415	0.054	0.335	0.008	<0.001	0.916	0.316

≤ 0.4 and ≥ 0.6 during the sunny and cloudy days, respectively (Fig. 2B). Changes in sky conditions from clear to cloudy tended also to change the other microclimatic parameters. On sunny days, air temperature (T_a ; Fig. 2C) and VPD (Fig. 2D) showed typical

daily courses, as characterized by their maximum and minimum values, but they remained more or less constant under the cloudy sky. Under the clear sky, daily maxima of T_a and VPD rose to ca 30.5 °C and 2.7 kPa, respectively, while these were only ca 17.8 °C

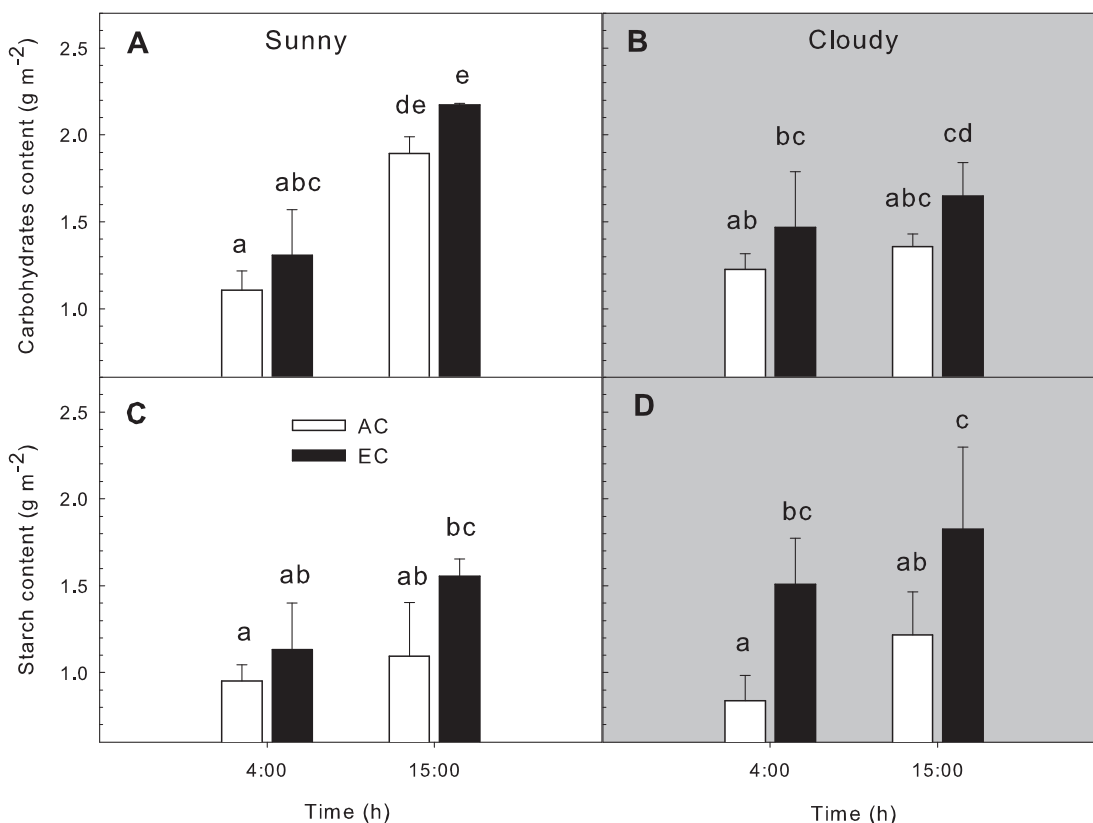


Fig. 3. Diurnal changes in contents of non-structural carbohydrates (A,B) and starch (C,D) expressed per leaf area unit under the conditions of sunny (A,C) and cloudy days (B,D). The measurements were done on the sun-adapted leaves of beech trees cultivated under ambient (AC; empty columns) and elevated (EC; full columns) CO₂ concentrations. The means (columns) and standard deviations (error bars) are presented ($N = 5$).

and 0.5 kPa under the cloudy sky. The average microclimatic conditions during the preceding two days for each of the two periods had been similar to those occurring during the measuring campaigns (data not shown). No statistically significant ($p > 0.05$) differences in PPFD, T_a or VPD were observed between the domes with AC versus EC.

3.2. Chemical composition of leaves

The EC treatment led to a slight increase in specific leaf mass (SLM), but no differences in nitrogen content per unit area (N_{area}) were observed between the AC and EC counterparts (Table 1).

Although the EC treatment led to decrease in total contents of chlorophylls (by 12–14%) and carotenoids (by 8–10%) per unit leaf area and to increase in the Chl a/b ratio (by ca 3%; Table 1), there were no significant interactions between CO_2 treatment and sky conditions (Table 2). The total content of xanthophyll cycle pigments (VAZ) per unit leaf area was not influenced by the EC treatment, and this amounted to 17–19 mg m⁻² irrespective of sky conditions (Table 1). The maximum de-epoxidation state of the xanthophyll cycle pigments (DEPS_{max}) was on average 71% and 64% under sunny and cloudy days, respectively. Under both sky conditions, the EC treatment led to higher DEPS_{max} values as compared to the AC treatment. These values were as much as 9% higher under clear sky ($p > 0.05$) and up to 14% higher under cloudy sky ($p < 0.05$) (Table 1).

Leaves treated under EC conditions had greater contents of non-structural carbohydrates (Fig. 3A,B) and starch (Fig. 3C,D) per unit leaf dry mass as compared to their AC counterparts, irrespective of sky conditions. However, most of these differences were

statistically non-significant (Table 2). Comparing morning (pre-dawn) and afternoon data, we observed an increase in afternoon non-structural carbohydrates of 69–72% during the sunny days, but it was only 16–20% during the cloudy days, irrespective of CO_2 treatment. Nevertheless, distinct dynamics in starch accumulation were observed between AC and EC plants. While the starch content increased in AC plants by only ca 25% during the sunny days, that increase was ca 150% during the cloudy days. In contrast, starch content increased by ca 40% in the EC plants during sunny days and by only ca 20% during cloudy days.

3.3. Dynamics of assimilation activity and stomatal conductance

Diurnal courses of A and G_s during sunny and cloudy days are shown in Fig. 4. Under clear sky conditions, significant stimulation of A by the EC treatment (33–37%) was found only during 10:00–12:00, while there were no differences in A values between treatments during the early morning and late afternoon hours (Fig. 4A), i.e. at PPFD ≤ 550 μmol (photons) m⁻² s⁻¹. Since the assimilation processes are limited by electron transport rate at low irradiances, identical courses of A under AC and EC had been expected under cloudy skies. However, significant reduction in A by as much as 47% was observed in EC plants as compared to AC plants during the afternoon hours (Fig. 4B). Except during early morning and late afternoon, EC treatment led to decreases in G_s under both sky conditions (Fig. 4C,D). Whereas the maximum G_s values were ca 35% lower in EC as compared to AC plants under clear sky conditions, under cloudy sky conditions that difference in G_s between AC and EC leaves was ca 72%. The stomata of EC plants remained almost closed throughout the

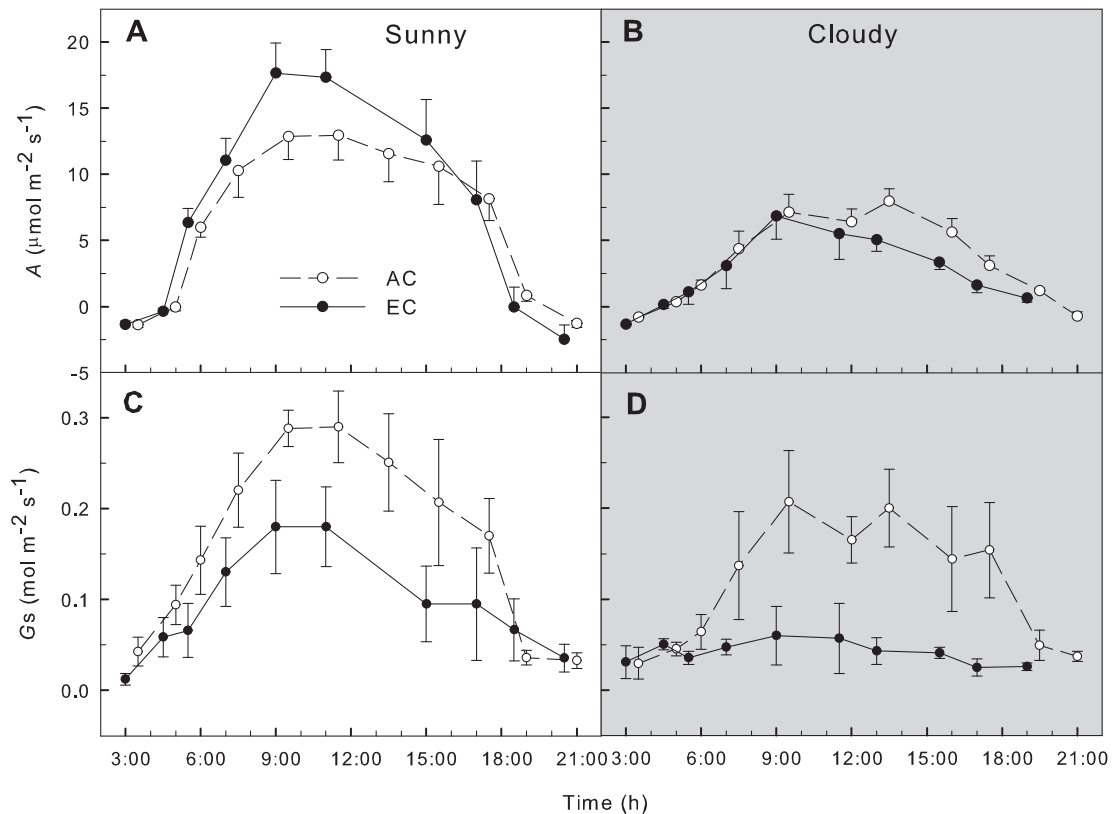


Fig. 4. Diurnal course of CO_2 assimilation rate (A) and stomatal conductance (G_s) under the conditions of sunny (A,C) and cloudy days (B,D). The measurements were done on the intact sun-adapted leaves of beech trees cultivated under ambient (AC; empty circles, dashed line) and elevated (EC; full circles, full line) CO_2 concentrations. The means (points) and standard deviations (error bars) are presented ($N = 3$).

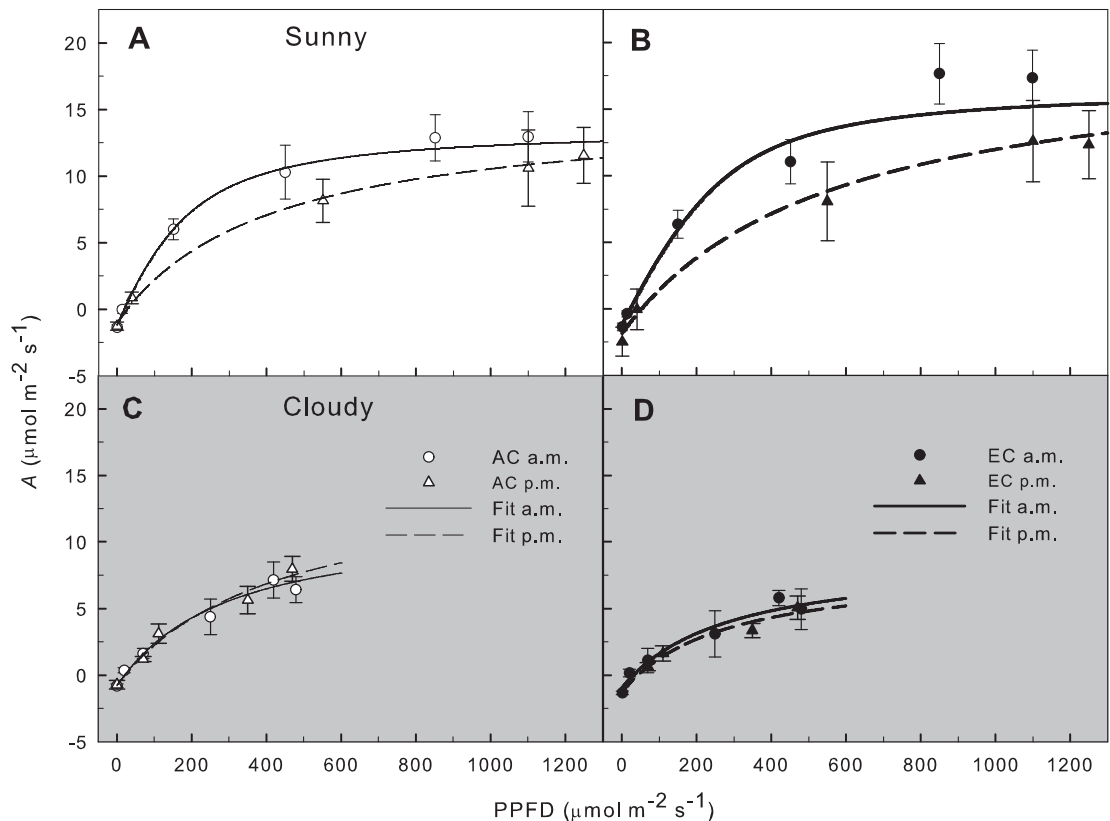


Fig. 5. Relationship between photosynthetic photon flux density (PPFD) and actual CO₂ assimilation rate (A) during sunny (A,B) and cloudy (C,D) days. The A–PPFD relationships estimated were divided into morning (00:00–12:00; circles, solid line) and afternoon (12:00–23:30; triangles, dashed line) parts. The nonrectangular hyperbolic function (Eqn. (3)) was fitted to the data ($R^2 = 0.84–0.93$; $p < 0.01$). Parameters of fitted light response curves are summarized in Table 3.

cloudy day, although the leaf-to-air vapour deficit (LAVD) ranged between 0.75 and 0.85 kPa irrespective of CO₂ treatment. Inter-cellular CO₂ was ca 320 and 580 μmol(CO₂) m^{−2} s^{−1}, respectively, during afternoon hours (12:00–16:00) of cloudy days in AC and EC conditions.

Parameters of the A–PPFD response curves (Fig. 5), derived from daily courses of A during clear and cloudy skies, are summarized in Table 3. During the sunny days, an afternoon depression of A in response to PPFD was evident for both CO₂ treatments. This depression in the afternoon was associated with decrease in the apparent quantum yield (AQY; by 38% for AC treatment and by 29% for EC treatment) and increase in the light compensation point (LCP; by 39% for AC treatment and 150% for EC treatment). By contrast, no afternoon depression occurred during the cloudy days under either CO₂ treatment.

To test whether the changes in G_s constitute the primary reason for the afternoon depression in carbon assimilation during sunny days, assimilation rate was plotted against conductance. Identical A–G_s relationships for both the sunny and cloudy days (Fig. 6) demonstrate that at leaf level the VPD effect overwhelms any other effects of clear versus cloudy sky.

To reveal the causes of reduced A under the EC treatment during cloudy sky conditions, analyses were made of EC-induced changes in light-saturated rates of Rubisco carboxylation *in vivo* (V_{Cmax}; Fig. 7), photorespiration rate (R_L; Fig. 8), and efficiency of primary photochemical reactions (Fig. 9). Significant decrease in V_{Cmax} induced by the EC treatment was found under actual (by as much as 40%; Fig. 7A,B) as well as normalized (as much as 55%; Fig. 7C,D) temperatures. Nevertheless, Rubisco carboxylation activity corrected to the leaf temperature of 25 °C (V_{Cmax,25}) reached daily

Table 3
Mean values ± standard deviations of selected parameters of CO₂ assimilation light response curves on the leaf-level (see fits in Fig. 5A,B) during sunny and cloudy days. Light response curves were divided into morning (00:00–12:00; a.m.) and afternoon (12:00–23:30; p.m.) parts. AC (EC) – ambient (elevated) CO₂ concentration, A_{max} – light saturated rate of CO₂ assimilation, AQY – apparent quantum yield, R_D – dark respiration rate, and LCP – light compensation. R² represents coefficient of determination.

		A _{max}	AQY	R _D	LCP	R ²
		μmol(CO ₂) m ^{−2} s ^{−1}	mol(CO ₂) mol(photon) ^{−1}	μmol(CO ₂) m ^{−2} s ^{−1}	μmol(photon) m ^{−2} s ^{−1}	–
Sunny	AC a.m.	14.8 ± 1.43	0.065 ± 0.0266	1.2 ± 0.18	18 ± 3.6	0.9313
	AC p.m.	16.2 ± 2.39	0.040 ± 0.0172	1.0 ± 0.21	25 ± 5.1	0.9051
	EC a.m.	17.8 ± 2.01	0.055 ± 0.0223	1.2 ± 0.31	22 ± 4.2	0.8555
	EC p.m.	21.5 ± 4.50	0.039 ± 0.0156	1.8 ± 0.40	55 ± 8.7	0.8759
Cloudy	AC a.m.	12.3 ± 1.30	0.041 ± 0.0114	0.8 ± 0.11	16 ± 3.1	0.9109
	AC p.m.	15.7 ± 1.20	0.037 ± 0.0087	1.7 ± 0.12	19 ± 4.0	0.9301
	EC a.m.	9.8 ± 2.60	0.034 ± 0.0152	0.9 ± 0.40	28 ± 5.6	0.8446
	EC p.m.	9.4 ± 1.11	0.034 ± 0.0080	1.3 ± 0.27	37 ± 7.4	0.9236

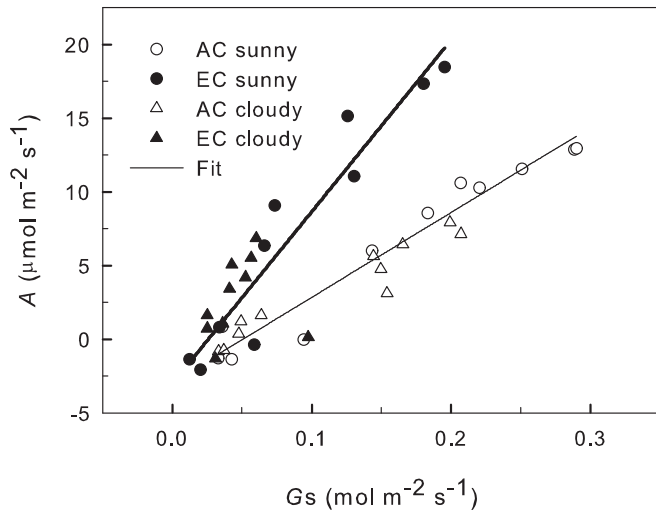


Fig. 6. Relationships between stomatal conductance (G_s) and CO_2 assimilation rate (A) estimated during sunny (circles) and cloudy (triangles) days. The measurements were done on the sun-adapted leaves of beech trees cultivated under ambient (AC; empty symbols) and elevated (EC; full symbols) CO_2 concentrations. The linear functions $y = 54.6x - 2.7$ ($R^2 = 0.94$; $p < 0.01$) and $y = 107.7x - 2.3$ ($R^2 = 0.80$; $p < 0.01$) were fitted to the relationships between A and G_s . ANCOVA revealed that CO_2 treatment had significant effect on the relationship ($p < 0.05$).

maxima of ca $45 \mu\text{mol}(\text{CO}_2) \text{m}^{-2} \text{s}^{-1}$ in AC plants irrespective of sky condition while significantly lower $V_{\text{Cmax},25}$ values were observed in EC plants under cloudy ($18.7 \mu\text{mol}(\text{CO}_2) \text{m}^{-2} \text{s}^{-1}$) as compared to clear ($31.7 \mu\text{mol}(\text{CO}_2) \text{m}^{-2} \text{s}^{-1}$; Fig. 7C,D) sky conditions.

Significance levels for the effects of $[\text{CO}_2]$ treatment, time of day, and sky conditions are summarized in Table 4.

As compared to clear sky conditions, cloudy sky led to marked reduction in R_L (Fig. 8), by 52–80% for AC and by 48–75% for EC. While the growth under EC resulted in 42–65% reduction of Rubisco oxygenation activity during the sunny days investigated, this reduction was only ca 33% during the cloudy days investigated. Noticeably, R_L values reveal no significant changes between AC and EC leaves during the midday hours (12:00–15:00) of cloudy days, which are those hours during which the significant reduction in A was observed in EC- as compared to AC-treated leaves (Fig. 4).

Irrespective of CO_2 treatment, the photochemical efficiency of PS II (Φ_{II}), estimated on the basis of chlorophyll fluorescence emission, did not decrease to below 0.3 and 0.5 during the sunny and cloudy days, respectively. In contrast to A , there were almost identical diurnal courses for the quantum yield of photosystem II photochemistry (Φ_{II} ; Fig. 9A,B) and the apparent photosynthetic electron transport rate (data not shown) as estimated under AC and EC conditions. However, a reduction of Φ_{II} , in accordance with the diurnal pattern of A , was observed during the midday hours (12:00–15:00) of cloudy days in EC as compared to AC leaves. The xanthophyll-dependent, non-photochemical dissipation processes within PS II (Φ_{NPQ} ; Fig. 9C,D) were higher in EC as compared to AC plants under clear (by 11% on average) and particularly under cloudy (by 33% on average) skies. Significance levels for the effects of $[\text{CO}_2]$ treatment, time of day, and sky conditions on fluorescence parameters are summarized in Table 4.

The total amount of fixed CO_2 and light use efficiency (LUE; defined as the ratio between the total amount of assimilated CO_2 and sum of photosynthetically active radiation incident on the leaf

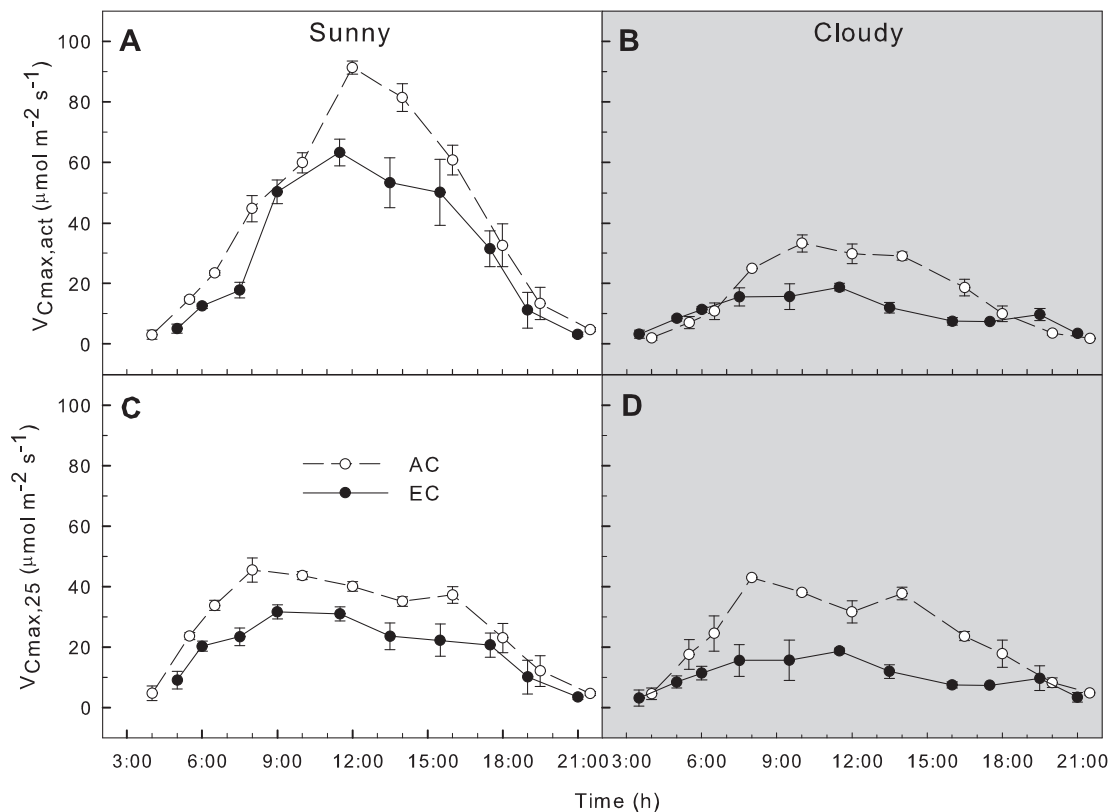


Fig. 7. Diurnal courses of *in vivo* light-saturated ($1400 \mu\text{mol}(\text{photons}) \text{m}^{-2} \text{s}^{-1}$) rates of Rubisco carboxylation (V_{max}) during sunny (A,C) and cloudy (B,D) days. The measurements were done on sun-adapted leaves of beech trees cultivated under ambient (AC; open circles) and elevated CO_2 concentration (EC; closed circles). The V_{max} values were estimated from carbon assimilation at actual leaf temperature ($V_{\text{max},\text{act}}$; A,B) and corrected to the leaf temperature of 25°C ($V_{\text{max},25}$; C,D) according to Bernacchi et al. (2001). Means (points) and standard deviations (error bars) are presented ($N = 3$).

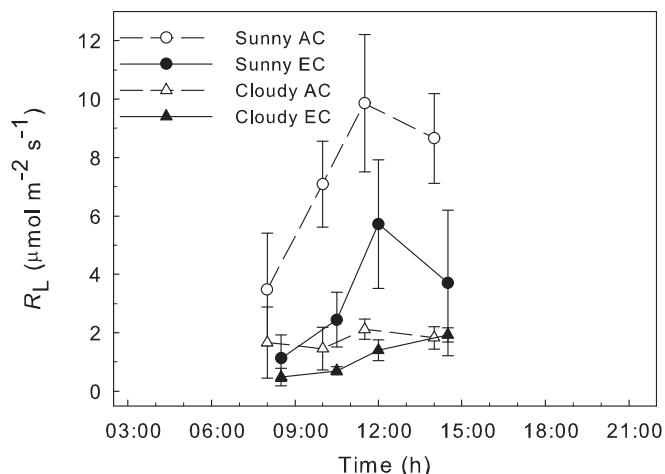


Fig. 8. Diurnal courses of photorespiration rate (R_L) during sunny (circles) and cloudy (triangles) days. The measurements were done on sun-adapted leaves of beech trees cultivated under ambient (AC; open symbols and dashed line) and elevated CO_2 concentration (EC; closed symbols and solid line). Means (points) and standard deviations (error bars) are presented ($N = 3$).

surface) was modelled for cloudy and sunny daytime periods (Table 5). The calculation was based on Equation (3) using daily courses of PPFD (Fig. 2) and the parameters of A –PPFD curves (Fig. 4). We found significantly greater carbon gain by sun-adapted leaves during sunny as compared to cloudy days (by 100% and 315% for AC and EC, respectively). Although the carbon gain of EC-treated

leaves was higher by 36% than in AC leaves for sunny days, it was lower by 34% on cloudy days. LUE increased by a significant 70% in AC-treated leaves under cloudy as compared to clear sky, whereas it was reduced by a slight 15% in EC-treated leaves.

4. Discussion

In accordance with many other researchers, we confirmed the stimulation of A under EC in *Fagus sylvatica* at irradiances above $550 \mu\text{mol (photons) m}^{-2} \text{s}^{-1}$ (Fig. 4), although the V_{Cmax} (Fig. 7) and Φ_{II} values (Fig. 9) were reduced by long-term EC treatment. We found that at leaf level EC increases the sum of daytime-fixed CO_2 and LUE under typical clear sky conditions, while both of these were reduced under cloudy sky compared to AC-grown plants (Table 5).

Cloud cover leads to a significant decrease in T_a and VPD daily maxima and increase in diffuse light fraction (Fig. 2). Brodersen et al. (2008) have proven that photosynthesis in sun-adapted leaves can be higher under direct light compared to equivalent irradiance of diffuse light. The columnar palisade cells are thought to facilitate the penetration of collimated direct light over isotropic diffuse light (Vogelman et al., 1996). On the contrary, the rate of photosynthesis in leaves at the top of canopies might be depressed during clear sky conditions because of stomatal closure, temperature-stimulated photorespiration, and/or photoinhibition at the highest irradiances (Kitao et al., 2006; Urban et al., 2012b). The A – G_s relationships (Fig. 6) demonstrate that at leaf level the VPD effect overwhelmed other effects of sky conditions of the days studied.

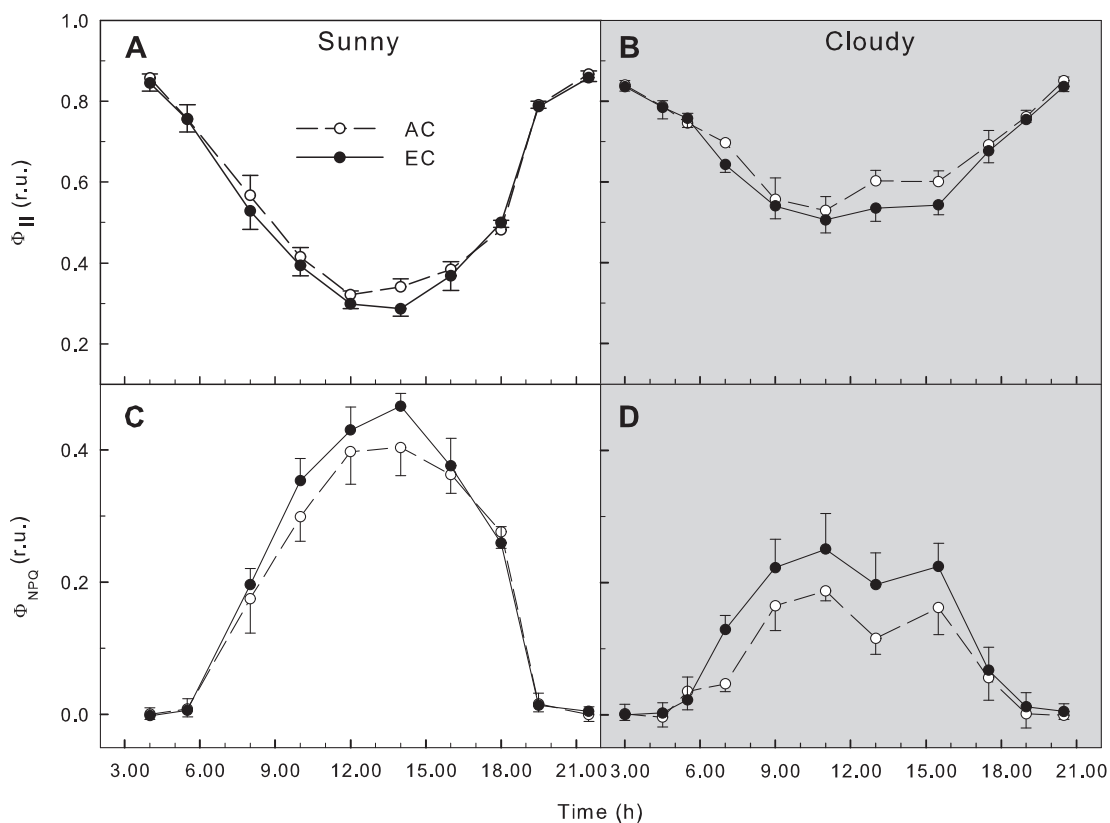


Fig. 9. Diurnal courses of actual quantum yield of photosystem (PS) II photochemistry (Φ_{II} ; A,B) and xanthophyll-dependent non-photochemical dissipation processes within the PS II (Φ_{NPQ} ; C,D) during sunny (A,C) and cloudy (B,D) days. The measurements were done on sun-adapted leaves of European beech (*Fagus sylvatica*) cultivated under ambient (AC; open symbols) and elevated CO_2 concentration (EC; closed symbols). Means (points) and standard deviations (error bars) are presented ($N = 3$).

Table 4

Summary of significance levels (p -values of three-way ANOVA) for the effects of CO₂ treatment, time of day, and sky conditions including mutual interactions on the parameters of carbon assimilation (A – CO₂ assimilation rate, G_s – stomatal conductance, $V_{C_{max,act}}$ – light-saturated rate of Rubisco carboxylation at actual leaf temperature, $V_{C_{max,25}}$ – light-saturated rate of Rubisco carboxylation corrected to the leaf temperature of 25 °C, R_L – photorespiration rate, Φ_{II} – actual quantum yield of photosystem II photochemistry, Φ_{NPQ} – xanthophyll-dependent non-photochemical dissipation processes within the PS II). Significant effects and interactions ($p < 0.05$) are indicated in bold.

	A	G_s	$V_{C_{max,act}}$	$V_{C_{max,25}}$	R_L	Φ_{II}	Φ_{NPQ}
Sky conditions	<0.001	<0.001	0.145	0.131	<0.001	<0.001	<0.001
Time	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
[CO ₂]	0.935	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Sky conditions × Time	<0.001	<0.001	<0.001	0.138	<0.001	<0.001	<0.001
Sky conditions × [CO ₂]	<0.001	0.645	0.079	0.059	<0.001	0.122	0.004
Time × [CO ₂]	0.003	<0.001	<0.001	0.152	0.672	<0.001	<0.001
Sky conditions × Time × [CO ₂]	<0.001	0.421	0.638	0.488	0.094	0.764	0.605

4.1. Clear sky conditions and EC

Since EC treatment usually leads to reduced transpiration water loss, we had hypothesized amelioration of the afternoon decline in photosynthesis in EC as compared with AC conditions during sunny days. Evidence in the literature relating to this hypothesis is contradictory. Although Spunda et al. (2005) indeed found lower midday decline in photosynthesis in EC-grown *Picea abies*, elevated [CO₂] also has been shown to have no impact on afternoon decline in photosynthesis in understory saplings (Singsaas et al., 2000) or in three poplar species grown in hot and dry climate conditions of central Italy (Bernacchi et al., 2003). Entirely contrary to this hypothesis, Kets et al. (2010) reported more pronounced midday decline in photosynthesis of *Populus tremuloides* grown in elevated compared to ambient [CO₂]. Based on the A –PPFD response curves (Fig. 5), we found pronounced afternoon depression of A at corresponding irradiances under EC relative to AC conditions, leading, among other things, to a large increase in the light compensation point (LCP; Table 3).

High photorespiration rates are associated with high PPFD and temperatures (Fig. 8). Reduction in G_s induced by EC (Fig. 4C,D) led to a subsequent increase in leaf temperature by as much as 4.5 °C as compared with AC leaves during the midday of sunny days (data not shown). Temperature-stimulated rates of photorespiration thus may contribute to enhanced CO₂ release and afternoon depression of carbon uptake in EC-treated leaves and plants during hot sunny days.

4.2. Cloudy sky conditions and EC

To the best of our knowledge, no comprehensive study has heretofore been undertaken on how elevated [CO₂] influences the diurnal pattern of photosynthesis under cloudy sky conditions. Since photons constitute the limiting substrate for photosynthesis under low, non-saturating irradiances, the rate of CO₂ assimilation is primarily limited by the regeneration of RuBP, which is to say by

ATP and NADPH production (Bernacchi et al., 2003; Sage, 1990). The electron transport rate – which is proportional to Φ_{II} (Fig. 9A,B) – was slightly reduced while Φ_{NPQ} was increased (Fig. 9C,D) by the EC treatment during cloudy days. We assume that this phenomenon is associated with EC-stimulated depoxidation of violaxanthin to zeaxanthin, which was observed particularly under the cloudy sky conditions (Tables 1 and 2). Enhanced DEPS increases thermal dissipation of solar radiation within PS II antennae (Eskling et al., 1997; Smirnoff, 1993), and that may consequently contribute to the flexible regulation of utilization of absorbed light energy, to ATP and NADPH production, and thus to the reduction of photosynthesis in the afternoon hours of cloudy days (Fig. 4B).

A conceptual model of the stomatal control by environmental factors predicts increase in G_s with high blue light intensity and low LAVD, corresponding to cloudy skies (Kanniah et al., 2013; Kets et al., 2010; Reinhardt and Smith, 2008; Urban et al., 2007), while it decreases with high intercellular [CO₂] and transpiration rate (Barillot et al., 2010). In the present study, the stomata of EC plants remained almost closed during the entire cloudy day, although the LAVD values were not influenced by [CO₂]. Similarly, Heath (1998) had found reduced G_s in *F. sylvatica* and *Castanea sativa*, but not in *Quercus robur*, grown at elevated [CO₂] on days with low VPD, while on warm sunny days (with correspondingly high VPD) G_s was unchanged or even slightly higher in elevated [CO₂] as compared to ambient [CO₂]. These findings may imply that plants grown in elevated [CO₂] require higher activation energy for stomatal opening and that low radiation energy from cloudy skies is unable to activate biochemical processes responsible for stomatal opening when [CO₂] is high. This is in agreement with a previous finding that accumulation of sucrose in the guard-cell wall strongly regulates stomatal aperture size in EC leaves and plants (Lu et al., 1997). However, there exists no data from direct microscopic observations of stomatal aperture in EC plants under cloudy sky conditions.

Notably, the accumulation of non-structural saccharides in cytosol was observed during clear sky conditions even as the concentration of starch in chloroplast stroma increased during cloudy sky conditions irrespective of [CO₂] treatment (Fig. 3), although some of these differences were not statistically significant (Table 2). We hypothesize that the main reason for starch accumulation during cloudy days is relatively low activity of such chloroplast transporters as triose-phosphate/phosphate translocator (TPT; Walters et al., 2004) that is caused by low temperature (Cho et al., 2012). Accordingly, accumulation of dihydroxyacetone phosphate in the chloroplasts leads to elevated starch biosynthesis during cloudy days. On the contrary, a rapid, TPT-supported transport of triose phosphates produced in photosynthesis to cytosol during hot sunny days results in stimulated biosynthesis of sucrose and other saccharides over starch (Fig. 3).

Table 5

The total amount of assimilated CO₂ (Carbon gain) and light use efficiency (LUE) of sun-adapted leaves of *Fagus sylvatica* over a day-time period (light intensity > 0 $\mu\text{mol (photon)} \text{ m}^{-2} \text{ s}^{-1}$) of the sunny and cloudy days. LUE has been calculated as the ratio between the total amount of assimilated CO₂ and sum of photosynthetically active radiation incident on the leaf surface. AC (EC) – ambient (elevated) CO₂ concentration.

		Carbon gain	LUE
		$\text{mol (CO}_2\text{)} \text{ m}^{-2} \text{ day}^{-1}$	$\text{mol (CO}_2\text{)} \text{ mol (photon)}^{-1}$
Sunny	AC	53.5	0.010
	EC	72.7	0.013
Cloudy	AC	26.7	0.017
	EC	17.5	0.011

5. Conclusions

We conclude that sky conditions have important effects on the relative impact of elevated $[CO_2]$ on photosynthesis and stomatal conductance and subsequently on the light use efficiency and total carbon gain. We can assume that an expected increase in cloud cover associated with climate warming (IPCC, 2007) may reduce the stimulatory effect of EC on leaf-level carbon uptake.

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